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Title

Cisplatin induces the release of extracellular vesicles from ovarian cancer cells that can induce invasiveness and drug resistance in bystander cells.

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Abstract

Ovarian cancer has a poor overall survival which is partly caused by resistance to drugs such as cisplatin. Resistance can be acquired as a result of changes to the tumour or due to altered interactions within the tumour microenvironment. Extracellular vesicles (EVs), small lipid-bound vesicles that are loaded with macromolecular cargo and released by cells, are emerging as mediators of communication in the tumour microenvironment. We previously showed that EVs mediate the bystander effect, a phenomenon in which stressed cells can communicate with neighbouring naïve cells leading to various effects including DNA damage; however, the role of EVs released following cisplatin treatment has not been tested. Here we show that treatment of cells with cisplatin led to the release of EVs that could induce invasion and increased resistance when taken up by bystander cells. This coincided with changes in p38 and JNK signalling, suggesting that these pathways may be involved in mediating the effects. We also show that EV uptake inhibitors could prevent this EV-mediated adaptive response and thus sensitise cells *in vitro* to the effects of cisplatin. Our results suggest that preventing pro-tumourigenic EV crosstalk during chemotherapy is a potential therapeutic target for improving outcome in ovarian cancer patients.

Keywords

Extracellular vesicles, exosomes, drug resistance, tumour microenvironment, bystander effect.

Intro

Ovarian cancer is the most fatal gynaecological cancer with more than 150,000 women succumbing to the disease each year worldwide (1). The five-year survival rate is less than 50% (2). Reasons for this high mortality rate include diagnosis at advanced stages and acquired resistance to chemotherapeutic drugs such as cisplatin and carboplatin (3-5). The causes for cisplatin resistance are complex and multifactorial (6-9). Along with numerous intracellular modulators, intercellular factors involving the tumour microenvironment have also been shown to play a crucial role in

cisplatin resistance; this can occur via altered communication between tumour cells and stromal cells (10), macrophages (11) or endothelial cells (12) as well as between tumour cells (13, 14).

In recent years, exosomes and microvesicles (collectively referred to as extracellular vesicles (EVs)) have gained prominence as mediators of intercellular communication. They have been shown to transfer RNAs and proteins that are functional in the recipient cells (15, 16). They can be found in various body fluids and in the tumour microenvironment and can transmit messages to neighbouring cells as well as to distant cells (17, 18). miRNAs, short non-coding RNAs whose levels can be altered during stress response (19), can also be shuttled between cells in the tumour microenvironment via EVs. Extracellular vesicles have been shown to modulate numerous factors in tumour cells including proliferation (20), viability (21) and metastatic capability (22). The tumour microenvironment has also been shown to be modulated by EV-mediated communication between the tumour and other cells such as cancer associated fibroblasts (23, 24) and mesenchymal stem cells (25). EVs are able to modulate angiogenesis, an important factor in cancer progression (26-29). EVs have also been shown to modulate the anti-tumoral response by affecting the immune response, T-cell activation and natural killer cell induction (30-33). EVs can also contribute to drug resistance via various mechanisms, including the sequestration of drugs (34, 35) and the transfer of proteins or RNA (36-39) (40-45). The morphology and the proteomic profile of EVs from multi-drug resistant tumours has been shown to be different from those from sensitive tumours (46) and EVs could be used as prognostic and diagnostic biomarkers in cancer (47).

Interestingly, EVs are involved in bystander effect (BE) (48). BE is a phenomenon in which stressed cells release soluble factors that when taken up by naïve recipient cells can induce phenotypic effects, including DNA damage (48-53). The potential roles for EVs released from cisplatin-stressed ovarian cancer cells, particularly in the context of the tumour microenvironment, have not been investigated. Here we show that chemotherapeutic treatment of ovarian tumour cells induces the release of EVs that can influence the phenotype of neighbouring naïve cells. Specifically, we show that the EVs released following cisplatin-stress response can induce increased invasiveness and drug-resistance in bystander cells. These effects coincided with changes in signalling via several pathways including p38 and JNK. Blocking the uptake of EVs during cisplatin treatment appeared to sensitise cells to the effect of the cytotoxic drug. Taken together these results suggest that EVs released by cells into the tumour microenvironment during chemotherapy could have an important role in mediating the progression of ovarian cancer.

Materials and Methods

Cell culture

All cell lines were maintained in RPMI media (Hyclone) supplemented with 10% foetal bovine serum (Gibco) and sub-cultured every 5-6 d using 0.05% trypsin with EDTA (Thermo Fisher Scientific). Cells were maintained at 37°C in a humidified incubator at 5% CO₂; fresh media was added every 2-3 d. A2780 and CP70 were kindly donated by Professor Robert Brown (University College London). IGROV-1 cells were purchased from National Cancer Institute, Frederick Cancer Division of Cancer Treatment and Diagnosis Tumor/Cell Line Repository (Bethesda, USA).

EV extraction

Foetal bovine serum was ultracentrifuged at 120 000 xg for 16 hours; RPMI or DMEM was then supplemented with 10% EV-depleted bovine serum to obtain EV-depleted media (EDM). Cells in T175 flasks at 70-80% confluence ($\sim 2.0 \times 10^7$) were grown overnight in EDM. For cisplatin treatments cells at 70% confluence were treated with a final concentration of 40 μ M cisplatin for 2 h at 37°C, cisplatin-containing media was removed, cells were washed with PBS, replenished with EDM and incubated for a further 2 h. After this time, media was removed to eliminate any cisplatin secreted by the treated cells and replenished with fresh EDM and this media was conditioned for 24 h. EVs were extracted from this conditioned medium by differential ultracentrifugation. Initially, it was centrifuged at 300 G for 5 min followed by centrifugation at 16,500 G for 20 min at 4°C. The media was then filtered using 0.22 μ m syringe filters blocked with 0.1% bovine serum albumin (BSA) (Sigma Aldrich). The supernatant was ultracentrifuged at 120,000 xg using Beckman Coulter Optima LE-80K ultracentrifuge for 90 mins at 4°C to pellet EVs. The extracted EVs were resuspended in PBS, and finally pelleted once more at 120,000 xg. EVs were resuspended in 50 μ L PBS and used in subsequent experimentation. When not used immediately after extraction EVs were stored at -80°C.

Western blotting of whole cell and EV protein extracts

Cells were scraped from the surface of a culture flask into ice cold PBS and pelleted at 300 G, washed with PBS and re-pelleted at 300 G. Cell preparations were then lysed in 1X radioimmunoprecipitation assay (RIPA) buffer (0.1 M Tris–hydrogen chloride, 0.3 M sodium chloride, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 1% Triton X 100) under constant agitation for 30 min at 4°C. Nuclei and cell debris were removed by centrifugation at 14,000 xg. For exosomal protein, EVs were resuspended in RIPA buffer and sonicated thrice for 5 min each in Decon FS100 frequency sweep sonicating water bath (Decon) with 15 sec of vortex mixing between each cycle with a final centrifugation at 14,000 G for 20 min at 4°C to pellet non-protein debris. Protein concentration was quantified by the BCA assay kit (Life Technologies). Approximately 10 μ g of cellular or exosomal protein was prepared in SDS–PAGE loading dye with dithiothreitol (DTT) and heated to 100 °C for 10 min. Samples were loaded onto a 12% denaturing polyacrylamide gel, electrophoresed and transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat dried milk powder (Marvel) in TBS–0.05% Tween (TBST) for 1 h at room temperature (RT) and then incubated overnight at 4°C with rabbit or mouse anti-human primary antibodies (Abcam) specific to HSP70 (ab5439) (EV marker) (1:2,000), cytochrome C oxidase (ab150422) (apoptotic body/mitochondrial marker) (1:1,700), GAPDH (ab128915) (cytoplasmic marker) (1:15,000), calnexin (ab22595) (endoplasmic reticulum marker) (1:120,000) and GM130 (ab31561) (Golgi marker) (1:1,000). Secondary anti-mouse Cy3- (Fisher) or anti-rabbit horseradish peroxidase (HRP)-tagged antibody (Abcam) (1: 2,000) incubations were then performed for 60 min at room temperature. Blots were digitally imaged for chemiluminescence with ECL solution (Bio-Rad) according to manufacturer's instructions or fluorescence for Cy3 using ChemiDoc MP (Bio-Rad).

Transmission electron microscopy of EV samples

A 12 μ L aliquot of each EV sample was combined with an equal volume of 4% paraformaldehyde (Sigma Aldrich) and incubated on ice for 15 min. A droplet of each sample was distributed using a pipette onto Parafilm (Thermo Fisher Scientific). Carbon-formvar coated copper 300 mesh grids (Agar Scientific, Stanstead) were placed dull-side downwards onto each sample droplet and left to incubate at room temperature for 30 min. Grids were then washed three times by placing dull-side downwards onto a droplet of 0.22 μ m filtered ultrapure water. Between each wash, excess water

was removed using filter paper. Finally, each grid was placed onto a 30 μ L droplet of 2% uranyl acetate (aqueous) (Sigma Aldrich) for 2 min. Excess solution was removed using filter paper and the samples were left to air dry for 60 min. Two grids were prepared from each aliquot. Grids were visualised using Hitachi H7650 Transmission Electron Microscope at 100 kV with x40,000 magnification. EV diameter was measured using the measurement function in AMT software (Advanced Microscopy Techniques, Massachusetts, USA).

EV size determination and quantification by Nanoparticle Tracking Analysis (NTA)

EV size and concentration were determined by NTA with a NanoSight LM10 instrument equipped with the NTA 2.0 analytical software (Malvern Instruments Ltd, Malvern). Five 30 sec videos of each sample were recorded and from these the software calculated the mean diameter (nm) and EV concentrations ($\times 10^8$ /ml). Each sample was measured in duplicate.

Matrigel transwell cell invasion assay

A2780 or IGROV-1 cells were treated with EVs extracted from cisplatin treated cells or untreated cells and starved of serum for 24 h prior to seeding in transwell inserts. The cells were harvested by trypsinisation and seeded at 100 000 cells/ well in Matrigel-coated 8 μ m pore membrane transwell inserts (BD Biosciences); a second dose of EVs was added into the respective wells at the same time. Complete medium supplemented with 10% FBS was loaded in the receiver wells to act as a chemoattractant. Cells were then incubated at 37°C for 24 h. The media was then removed from the inserts and the upper surface of the inserts swabbed with a cotton bud to remove any cells that had not invaded the membrane. The inserts were then washed with PBS and stained with 1% crystal violet (Sigma Aldrich) for 10 min, washed again with distilled water and mounted onto glass slides using di-N-butyle phthalate in xylene (DEPEX) and glass coverslips. The membranes were visualised using a Zeiss Axioplan inverted microscope using x125 magnification in differential interference contrast and all the cells were counted.

Proteome Profiler

EVs derived from control cells or cisplatin treated cells were added to fresh A2780 cells (EVs derived from 1 T175 flask to 1 $\times 10^6$ cells) and incubated for 24 h. The Proteome Profiler Human PhosPho-MAPK Array (R&D Systems, Abingdon) array procedure was performed as outlined in the manufacturer's protocol.

Effect of EVs on cell viability and cisplatin response

A2780 cells were seeded into 96 well plates (day 0) at 10 000 cells per well. 24 h later (day 1), cells were treated with EVs extracted from cisplatin-treated or untreated A2780 cells or PBS. On day 2, half of the wells in all groups were treated with 20 μ M cisplatin (Fisher; stock solution - 16.7 mM made up in PBS) for 3 h diluted in media. For experiments with heparin, cells were pre-treated with 10 μ g/ml heparin (Sigma, H3393) diluted in media for 30 min prior to cisplatin treatment at a final concentration of 20 μ M. 3 h after commencement of cisplatin treatment, the media containing cisplatin was removed, cells were washed with PBS and fresh media was added; heparin treatment was continued for cells in the heparin-treatment group. On day 4, MTT assay was performed as previously described (54, 55) to assess the viability of the cells in each group.

Effect of EV uptake inhibitors on cisplatin response

Cells were seeded in 96 well plates at 6,000, 10,000 and 15,000 cells per well for CP70, A2780 and IGROV-1 cells respectively. 48 h later, cells were pre-treated with EV uptake inhibitor as follows.

Heparin (Sigma Aldrich, H3393) was diluted to a concentration of 10 mg/ml in deionised distilled water, filtered through 0.22 µm filters and stored at -20°C; it was diluted in media and added to cells at a final concentration of 10 µg/ml. Amiloride (5-(N-ethyl-N-isopropyl) amiloride, or EIPA) (Sigma Aldrich, A3085) was stored in DMSO at a concentration of 108 mM and added to cells at 50 µM concentration. Dynasore (Sigma, D7693) was diluted in DMSO to a concentration of 31 mM, stored at -20°C and added to cells at a concentration of 50 µM. 30 min after the drug treatment, cisplatin was added at varying concentrations to give a cisplatin response curve. Three hours later, cisplatin was removed, cells were washed with PBS and fresh media added; inhibitors were added to the same wells as before. An MTT assay was carried out 48 h after cisplatin treatment to assess the viability of cells in each group.

Xenograft experiments

Xenograft experiments were carried out at the animal testing facility at Queen's University, Belfast by Prof Helen McCarthy; A2780s for this experiment were kindly provided by Dr Fiona Furlong, Queen's University, Belfast. Five million A2780 cells in matrigel were implanted subcutaneously into the flanks of BALB/c SCID mice. Animals were monitored regularly and body weights were measured three times a week. Tumour volume was calculated using the formula

$$V = \frac{4}{3}\pi r^3$$

Where r is half of the geometric mean diameter (GMD), calculated as

$$\sqrt[3]{L*B*D}$$

Treatment was started when the tumour measured 100 mm³. Twenty four mice were then divided into four treatment groups (1) cisplatin 5mg/kg once weekly i.p. (2) Heparin (Sigma, H3393) only – 10 mg/kg every day i.p. (3) combination group – cisplatin 5mg/kg once weekly i.p. and heparin 10 mg/kg once daily i.p. and (4) control group. Tumour volume was monitored three times a week; when the tumour quadrupled in size, the animal was sacrificed. Any mice that lost 20% of body weight during the experiment were sacrificed as the treatment was deemed too toxic. All animal experiments were performed in adherence to our home office license (PPL2678).

Statistical Analysis

The Student's T-test was used to determine statistical significance unless otherwise stated. GraphPad Prism was used to calculate IC50s for curves and to analyse significance in differences between IC50s of curves using the extra sum-of-squares F test. For all experiments at least three biological replicates for each point were performed to enable statistical comparisons. P-values in figures are depicted as follows: <0.05 - *, <0.01 - **, <0.001 - ***.

Results and discussion

Characteristics of EVs from control and cisplatin treated cells

As a first step in assessing the role of EVs in the tumour microenvironment following drug treatment we compared the characteristics of EVs from control cells (control EVs) and those from cells treated with cisplatin (cisplatin EVs). EVs were extracted by ultracentrifugation of media conditioned overnight with A2780 control cells or A2780 cells treated with cisplatin. Western blotting (figure 1A) confirmed the presence of GAPDH and HSP70 which are known to be present in EVs; the absence of

calnexin and cytochrome C in both EV pellets established the absence of cellular contamination in the EV preparations. Total particle number was estimated by nanoparticle tracking analysis. There was no significant difference between the concentration of control EVs (13.0×10^8 particles/mL) and cisplatin EVs (17.2×10^8 particles/mL) (figure 1B). Some groups have reported increases in EV-production following various types of stress (49, 56-58); however, in our hands we observe small but non-significant increases in EV release following cisplatin (present work) and heat stress (59). This discrepancy could be due to experimental difference such as doses of treatment or the period of EV conditioning. Nevertheless, our results confirm the presence of EVs released by cancer cells following treatment.

To further characterise EVs from the tumour cells we performed transmission electron microscopy (TEM). TEM of the EVs from both control and cisplatin-treated cells showed vesicles of the expected size and morphology (figure 1C), with diameters ranging from 30nm to 160nm; however, the mean diameter of the cisplatin EVs (49.3 nm) was significantly smaller than that of the control EVs (87.6 nm) (student's t-test $p = 2.1 \times 10^{-8}$) (figure 1D). Interestingly, we also find that TEM measurements of EVs released following heat (59) are smaller compared to those released under normal conditions. That the imaged EVs from stressed cells appear smaller could represent a genuine difference in size, or could represent a different biophysical property that causes an artefact of preparation in the TEM that leads to the EVs appearing smaller. Nevertheless, the results suggest there are qualitative differences between EVs that are released following cisplatin treatment, and is consistent with studies that have also demonstrated differences in the content of EVs that are released under conditions of stress (57, 58, 60-62).

EVs released by cisplatin-treated cells have the capacity to induce invasion

We hypothesise that treatment of cells with cytotoxic compounds such as cisplatin leads to the release of EVs into the tumour microenvironment with the capacity to influence other tumour cells. To test the effect of cisplatin EVs on invasiveness of A2780s we used the matrigel invasion assay. Treatment with cisplatin EVs increased the invasiveness of A2780s by approximately 6-fold compared to A2780s treated with control EVs ($p = 0.0082$) (figure 2A). Similar results were obtained from IGROV-1, with cisplatin EVs increasing invasiveness by about 5-fold ($p = 0.042$) (figure 2B). Various studies have shown that EVs can induce an invasive and motile phenotype in recipient cells (63-65). Our results suggest that, at least in the case of A2780 and IGROV1 cells, the EVs released under normal conditions cannot induce greater levels of invasion when added to cells. However, the qualitative changes in EVs induced by cisplatin treatment confer the ability to induce greater invasion. Interestingly our unpublished data also show that EVs released following heat stress can also induce greater invasion in recipient cells(59), and others have shown that EVs released following exposure of cells to ionising radiation can induce increased metastatic ability (57). These results suggest that the release of EVs with the ability to induce invasiveness in recipient cells may be part of a more general intercellular response to stress which could be occurring in the tumour microenvironment.

Cisplatin EVs can cause bystander effect and an adaptive response to cisplatin

The bystander effect (BE) is a phenomenon in which stressed cells can communicate with other cells, leading to apparently detrimental effects such as DNA damage in these bystander cells (48-50, 52, 53, 66). Our recent work showed that EVs mediate BE following irradiation (48, 67). Subsequent findings have confirmed that EVs mediate BE following different stresses, including radiation (48, 49,

67) and heat shock(59). We therefore hypothesised that cancer cells stressed by the addition of cytotoxic chemotherapeutics could release EVs into the tumour microenvironment, which could then be taken up by other cells (including other cancer cells) leading to potential effects on tumour progression.

To test the hypothesis that EVs released by stressed ovarian cancer cells could modulate the activity of bystander cells we studied the effect of cisplatin on A2780 cells. EVs derived from cisplatin-treated cells appear to decrease the viability of bystander A2780 cells by 10% compared to cells treated with EVs from unstressed cells ($p = 0.027$) (figure 3A). Heparin has been shown to inhibit uptake of EVs (68, 69). Treating bystander cells with heparin, which in our hands also inhibits EV uptake by >95% (data not shown), abrogates the ability of cisplatin-EVs to mediate BE (figure 3A). These results are consistent with previous findings showing that cytotoxic agents including mitomycin C (53, 66, 70), bleomycin (66, 71) and vincristine (53) can induce BE. Our findings, which to our knowledge are the first to reveal an EV-mediated bystander effect induced by cisplatin, support the hypothesis that a wide range of stressors can induce the release of EVs into the tumour microenvironment that can potentially modulate an inter-cellular stress response in a tumour.

The BE has been observed in several species, suggesting it provides a beneficial effect that has been conserved through evolution (72-74). One consequence of BE is an adaptive response which renders bystander cells more resistant to future stressors (52). Indeed, we have observed that bystander cells that take up EVs released from heat-shocked cells are more resistant to a repeated dose of heat-shock (59). To test whether a similar effect occurs in ovarian cancer cells following treatment with chemotherapeutics, we performed EV-mediated BE as described above, treated these bystander cells with a dose of cisplatin and by performing an MTT assay on these cells we effectively measured the effect of EV treatment on their resistance to cisplatin. When cells were pre-treated with cisplatin-EVs and then challenged with cisplatin they were significantly protected compared to cells pre-treated with PBS (figure 3B). This protective effect was reduced (but not completely abrogated, $p = 0.052$) when the cells were also treated with heparin to block the uptake of the EVs (figure 3B). The observation that heparin does not completely block this adaptive response could be because either additional non-vesicular factors are required for the response, or because some of the effects could be mediated by interaction of the EV with receptors at the cell surface without the need for EV uptake and cargo delivery. A recent study has also demonstrated that EVs released following treatment of pancreatic cancer cells with gemcitabine can induce an adaptive response in recipient cells, which may be mediated by enhanced reactive oxygen species detoxification and miR-155-induced suppression of a gemcitabine metabolising enzyme (75). Taken together, our results are consistent with the hypothesis that treatment of cells with cisplatin- EVs induces a survival mechanism that allows them to adapt to resist the effects of cisplatin.

Pathways differentially modulated by cisplatin EVs in cells

To investigate the potential mechanisms by which cisplatin-EVs induce bystander effects (including increased invasiveness and the adaptive response) we analysed changes in phosphorylation status of important signalling proteins. A2780 cells were treated with cisplatin-EVs or control-EVs, proteins were extracted and changes in phosphorylation were measured using a Phospho-MAPK Array (figure 4). Seven proteins had significantly different relative levels of phosphorylation between the cells treated with cisplatin EVs compared with control EV. CREB ($p = 0.019$), extracellular signal-regulated kinases (ERK) 2 ($p = 0.050$) and TOR ($p = 0.0021$) kinases were down-regulated in A2780s treated

with cisplatin EVs, whilst JNK2 ($p = 0.00087$), JNK-pan ($p = 0.044$), p38 α ($p = 0.038$) and p53 ($p = 0.015$) were up-regulated. These results suggest the involvement of these pathways in the effects specifically mediated by cisplatin EVs, some of which are consistent with previously implicated roles in cisplatin resistance. Knockdown of p38, for example, can lead to increased sensitivity to cisplatin (76), suggesting that the activation of p38 we observe in our cells could lead to increased resistance and therefore an adaptive response. JNK signaling is known to be involved in stress response and JNK phosphorylation is activated by a variety of stressors (77). In the context of cisplatin treatment JNK may be a 'double-edged sword', in that it can have pro-apoptotic effects but can also be associated cisplatin resistance (78). These context-dependent effects may explain the observation that bystander cells appear to be simultaneously more stressed and more protected against future stress. Interestingly, JNK activation can also enhance the invasive and migratory behavior of cells (79), suggesting that this may also underlie our observation that EVs released following cisplatin-mediated stress induce greater invasion. Additional experiments are needed to elucidate whether delivery of vesicular cargo is required, whether signalling changes are responsible for the increased invasion, and whether increased vesicular delivery of matrix metalloproteinases to the extracellular environment is an important factor. Taken together our results suggest that changes in signalling activity are associated with the range of EV-mediated bystander effects observed following cisplatin treatment. Further work is needed to assess whether these effects occur within the tumour microenvironment and how these could contribute to tumour progression *in vivo*.

EV uptake inhibitors sensitise cells to cisplatin

The results above indicate that EVs released by cisplatin-treated cells can induce an adaptive response in bystander cells. When treating a population of cells with cisplatin it would be difficult to disentangle the direct effect of the drug from any bystander effects that may be occurring; nevertheless, we reasoned that when such a cohort of cells is undergoing stress the transfer of cisplatin-EVs will occur, which could lead to the invasive and adaptive response we observe in bystander cells. If this is the case then the EV-mediated communication during cisplatin treatment would help the population to become more resistant to the drug. We therefore hypothesised that inhibiting this communication using heparin as an EV uptake blocker should prevent the adaptive response and thus sensitise the cells to the effects of cisplatin. To test this hypothesis, ovarian cancer cell lines were treated with EV uptake inhibitors and cisplatin and the effects on overall survival were measured using the MTT assay. Treatment of cells with heparin alone did not reduce overall survival of cells (data not shown), suggesting that heparin itself was not toxic at the doses used. Interestingly, pre-treatment of cells with heparin significantly decreased the IC₅₀ of A2780 cells from 31.3 μ M to 21.2 μ M ($p < 0.0001$) (figure 5A). Similar results were obtained in IGROV-1 (figure 5B) and CP70 (figure 5C) cell lines with the IC₅₀ decreasing from 60.4 μ M and 146.8 μ M to 52.6 μ M and 118.9 μ M, respectively ($p < 0.0001$). To test whether other EV uptake inhibitors also have the same cisplatin-sensitising effect we used amiloride and dynasore, which inhibit micropinocytosis and dynamin-requiring uptake pathways, respectively (80). As expected, amiloride and dynasore both significantly sensitised A2780 cells to cisplatin (figure 5D). These results suggest that blocking EV transfer between cells in the tumour microenvironment during chemotherapy could lead to more effective killing of cancer cells.

Interestingly, heparin has previously been shown to increase sensitivity to cisplatin. In a recent study tinzaparin, a low-molecular weight heparin, was shown to sensitise A2780 cells to cisplatin, and that

cell surface heparan sulfate proteoglycans were involved (81). Heparin has also been shown to decrease invasion and migration in breast and lung cancer cell lines (82-84). Heparin was also shown to increase cytotoxicity caused by chemotherapeutic drugs in breast cancer cells; an effect on drug efflux transporters ABCG2 and ABCC1 was noted that led to increasing levels of cytotoxic drugs within cells (85). Our results suggest that sensitising effect of heparin may be at least in part due to inhibiting the EV-mediated cross-talk between cells during treatment.

Heparin does not appear to increase cisplatin sensitivity *in vivo*

The finding that treating ovarian cancer cells with heparin sensitises them to cisplatin has obvious therapeutic implications. To test whether heparin could sensitise ovarian cancer cells *in vivo* we utilised a xenograft model. A2780 cells were implanted subcutaneously in the flank of BALB/c SCID mice. The mice were then assigned to one of four groups a) control b) cisplatin treatment only c) heparin treatment only and d) heparin and cisplatin treatment; tumour volume was regularly monitored to test the effect of treatment on tumour progression. Tumour doubling-time, volume, and survival are shown in figures 6A, 6B and 6C, respectively. Contrary to our expectations, heparin when given alongside cisplatin did not slow the growth of the tumour nor did it decrease the survival. Indeed, the addition of heparin appeared to speed up doubling time compared to adding cisplatin alone. Thus, it appears from this experiment that either heparin does not have the same effect on EV communication *in vivo*, or that the drug has other pro-tumour side-effects in the xenograft setting that negate any beneficial effects.

The discrepancy between *in vitro* and *in vivo* results could be due to the experimental conditions being used. The xenografts, for example, were injected into flanks and may not represent the best possible model for studying behaviour of ovarian cancer *in situ*. Heparin could also help tumour growth by other means, for example by inducing a higher degree of angiogenesis (86). Results from other xenograft studies that investigated the effect of xenograft sensitivity to drugs appear to be at odds with our own data. In one study, both tinzaparin (a low molecular weight heparin) and a non-anticoagulant heparin, S-NACH, decreased tumour growth and increased apoptosis in pancreatic cancer cells (87, 88); S-NACH also increased chemotherapy sensitivity in breast cancer xenografts (89). Xenograft studies appear to suggest that heparin decreased tumour growth in lung cancer (90) and to confer sensitivity to gefitinib, a chemotherapeutic agent (91). Another study revealed that heparin decreases cisplatin resistance of lung cancer cells (92). However other studies appear to suggest no benefit of adding heparin to conventional chemotherapy (93-95). The differences observed in these disparate studies may be down to subtle differences in methodology or the study model. Future experiments should be performed to ascertain the potential of heparin, and other EV uptake inhibitors in sensitising cancer cells to drug treatment.

Conclusion

Here we have demonstrated that EVs released following treatment of cells with a cisplatin are able to induce a range of effects in recipient cells. These include an adaptive response which yields greater resistance to drug treatment, and increased invasiveness. Blocking the transfer of EVs between cells may represent a means to sensitise tumours to chemotherapy, though further work is needed to establish whether these benefits can be translated to the *in vivo* setting. Future work is also needed to fully elucidate the pathways involved and how they contribute to tumour progression. These data further highlight the importance of intercellular communication via EVs in the tumour microenvironment.

Ethics

All animal experiments were performed at the animal testing facility at Queen's University, Belfast in adherence to our home office license (PPL2678).

Competing Interests

The authors state that we have no competing interests.

Authors contributions

Conceived project – DRFC; conceived and designed experiments – DRFC, PS, LAM, SB, RCP; performed experiments– PS, LAM, FF, HOM; analysed data – DRFC, PS, LAM; supervision of work – DRFC, SB, RCP; wrote the paper – DRFC, PS; critically reviewed manuscript – DRFC, PS, LAM, SB, RCP, FF, HOM.

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Figures and figure legends

Figure 1 Characterisation of cisplatin and control EVs derived from A2780 cells A) Control and cisplatin treated cell and EV protein lysates were characterised by Western blotting, samples were probed for GAPDH, Calnexin, HSP70 and Cytochrome C Oxidase. B) Quantification of EVs secreted by control and cisplatin treated A2780 cells by nanoparticle tracking analysis (at least two replicates). C) Images of electron microscopy grids of control and cisplatin EVs visualised by transmission electron microscopy. D) Average diameter of EVs secreted by cisplatin treated and control A2780 cells measured on electron microscopy grids (C).

Figure 2 The effect of cisplatin treated cell-derived EVs upon the invasive capacity of ovarian cancer cell lines The Matrigel transwell invasion assay was used to determine the effect of cisplatin treated cell-derived EVs on invasive potential of two ovarian cancer cell lines, A2780 (A) and IGROV-1 (B). Extracted EVs were administered to approximately 1 million cells and after 24 h 100,000 cells were distributed into each insert of the transwell assay and another dose of EVs was added. After 24 h the Matrigel membranes were cleared of non-invasive cells and invasive cells were stained with crystal violet. The number of invasive cells on each membrane was counted. The graphs represent fold change in terms of the total number of cells that invaded the Matrigel membrane following treatment with either control or cisplatin treated cell-derived EVs. Each sample group contained six biological replicates. Error bars represent standard error of the mean of the biological replicates. P values were calculated using T-test. Representative images are shown below each group.

Figure 3 Cisplatin derived EVs cause bystander effect and an adaptive response to cisplatin

A. A2780 cells were seeded in 96-well plates. They were treated with PBS (control), EVs from cisplatin-treated cells (cis EV) or EVs from control cells (control EV) with or without 30 minutes pre-treatment with 10µg/ml heparin to inhibit EV uptake. 4 d later overall viability was measured using the MTT assay. Cis EVs caused a significant decrease in viability; this effect was not present in the group treated with heparin. B. A2780 cells were treated with PBS, Cis EVs or Control EV (as in panel

A) with or without pre-treatment with heparin (10µg/ml); after 24 h cells were further treated with cisplatin and survival was assayed using the MTT assay (results are normalised to control). Cells pre-treated with cis EVs are more resistant to cisplatin; this effect is decreased with heparin. Each column shows the mean of at least 6 replicates; error bars show standard error of mean.

Figure 4 Relative phosphorylation levels of 26 proteins in A2780 cells following treatment with either control or cisplatin EVs determined using the Proteome Profiler Human Phospho-MAPK Array A) Blots showing intensity for each kinase on duplicate spots for each EV treatment. B) Intensity levels of each kinase in A-2780 cells treated with either control or cisplatin EVs. Differences in kinase phosphorylation were calculated using the two tailed T-test.

Figure 5 EV inhibitors alter the cisplatin sensitivity of ovarian cancer cell lines

Cells were seeded in 96-well plates (day 0); they were pre-treated on day 2 for 30 min with heparin (panels A, B and C), amiloride or dynasore (panel D). They were then treated with varying concentrations of cisplatin for 3 h. Viability was quantified by the MTT assay after 48 h. There was a significant increase in sensitivity to cisplatin in A2780 (A) ($p < 0.0001$), IGROV-1 (B) ($p = 0.0006$) and CP70 (C) ($p < 0.0001$) on pre-treatment with heparin. Similarly, there was an increased sensitivity to cisplatin in A2780 cells on pre-treatment with amiloride (D) ($p < 0.0001$) and dynasore (D) ($p < 0.0001$)

Figure 6 The effect of heparin on cisplatin treatment in A2780 xenografts in mice

A2780s were injected subcutaneously into the flanks of nude BALB/c SCID mice; when the tumours were 100 mm³ in volume 24 mice were divided into four treatment groups - control (no treatment), heparin only, cisplatin only or heparin and cisplatin. Tumour volume was assessed regularly; the animal was sacrificed when the tumour quadrupled in size. Panel A shows tumour doubling times in the four groups; panel B shows average tumour volume in the four treatment groups while the Kaplan-Meier curve in Panel C shows survival after treatment.